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(54) Title: ANTICOAGULANT PEPTIDE ALDEHYDE DERIVATIVES

(57) Abstract

This invention relates to new peptide aldehyde derivatives of general formula (I): D-Xaa-Pro-Arg-H, wherein Xaa represents a 2-cycloheptyl-2-hydroxyacetyl or 2-cyclopentyl-2-hydroxyacetyl group, Pro represents an L-prolyl residue and Arg represents an L-arginyl residue, their acid-addition salts formed with an organic or inorganic acid and pharmaceutical compositions containing the same. The compounds of general formula (I) of the invention have valuable therapeutic, particularly anticoagulant, antiplatelet and antithrombotic, properties.

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ANTICOAGULANT PEPTIDE ALDEHYDE DERIVATIVES

This invention relates to new peptide aldehyde derivatives of general formula (I),

D-Xaa-Pro-Arg-H (I)

wherein

Xaa represents a 2-cycloheptyl-2-hydroxyacetyl or 2-cyclopentyl-2-hydroxyacetyl group,

Pro represents an L-prolyl residue and

Arg represents an L-arginyl residue,

their acid-addition salts formed with an organic or inorganic acid and pharmaceutical compositions containing the same.

The compounds of general formula (I) of the invention have valuable therapeutic, particularly anticoagulant, antiplatelet and antithrombotic, properties.

Particularly preferred representatives of the compounds of general formula (I) of the invention are the following derivatives:

D-2-cycloheptyl-2-hydroxyacetyl-L-prolyl-L-arginine aldehyde hemisulfate and

D-2-cyclopentyl-2-hydroxyacetyl-L-prolyl-L-arginine aldehyde hemisulfate.

Definitions

The abbreviations of the hydroxy- and aminoacids, their substituents and peptides built up therefrom are in accordance with the prior art, e. g. Biochem. J. **126**, 773 (1972); Biochemistry **14**, 449 (1975).

Amino acids:

Arg = L-arginine [(2R)-2-amino-5-guanidino-pentanoic acid],

Asp = L-aspartic acid [(2S)-2-amino-3-carboxypropionic acid],

boroArg = L-boroarginine [(1R)-1-amino-4-guanidino-butylboric acid],

D-Chg = D-2-cyclohexylglycine [(2R)-2-amino-cyclohexyl-acetic acid],

D-cHga = D-2-cycloheptyl-2-hydroxyacetic acid [(2R)-2--cycloheptyl-2-hydroxyacetic acid],

D-cPga = D-2-cyclopentyl-2-hydroxyacetic acid [(2R)-2--cyclopentyl-2-hydroxyacetic acid],

Gla = gamma-carboxy-L-glutamic acid [(2S)-2-amino-4,4--dicarboxybutyric acid],

Glu = L-glutamic acid [(2S)-2-amino-4-carboxybutyric acid],

Gly = glycine (2-aminoacetic acid),

D-Hma = hexahydro-D-amygdalic acid [(2R)-2-cyclohexyl-2--hydroxyacetic acid],

D-MePhe = N-methyl-3-phenyl-D-alanine [(2R)-2-methyl-amino-3-phenylpropionic acid],

D-MePhg = D-N-methylphenylglycine [(2R)-2-amino-2--phenylacetic acid],

Nal(1) = 3-(naphth-1-yl)-L-alanine [(2S)-2-amino-3-(naphth--1-yl)-propionic acid],

D-Phe = 3-phenyl-D-alanine [(2R)-2-amino-3-phenylpropionic acid],

Pro = L-proline [(2S)-pyrrolidine-2-carboxylic acid].

Substituents:

Ac = acetyl, Boc = tert-butoxycarbonyl, Bz = benzoyl, Bzl = benzyl, Me = methyl, 4MeP = 4-methyl-pentanoyl, pNA = p-nitrophenylamino, THP = tetrahydro-pyranyl, Tos = p-toluenesulfonyl, Z = benzyloxycarbonyl.

Peptides and derivatives:

The abbreviations of amino acids alone represent the respective L-amino acid. The D-amino acid is marked separately, e. g. 3-phenyl-D-alanine = D-Phe. The hyphen before and after the amino acid abbreviation means a missing hydrogen atom from the amino group or a missing hydroxy group from the carboxy group, resp. Accordingly, D-cHga-Pro-Arg-H represents D-cycloheptyl-2-hydroxy-acetyl-L-prolyl-L-arginine aldehyde and Bz-Ile-Glu-Gly-Arg--pNA represents benzoyl-L-isoleucyl-L-glutamyl-glycyl-L--arginine p-nitroanilide.

Description of the known art

Blood clotting represents part of the protective mechanism in the organism. Vessel wall injury initiates the cascade, a blood clot is formed to inhibit bleeding to death. In addition, vascular diseases, haemostasis and pathological activation of clotting factors may also induce blood clotting. The vessel is obstructed fully or partially by the intravascular thrombus formed and thrombosis develops. Fibrinolysis represents an other part of the protective mechanism. Here excess blood clots are removed by the

enzymes participating in thrombolysis and the dissolution of the thrombus, too.

The blood clotting process is a cascade reaction, a series of catalyzed enzyme reactions, where plasma proteins, i. e. the clotting factors, are activated consecutively. The factors are designated by Roman figures, the active form is represented by the letter "a". Trivial names are in use too, thus fibrinogen = factor I (fI), fibrin = factor Ia (fIa), prothrombin = factor II (fII) and thrombin = factor IIa (fIIa). Serine proteases (fXIIa, fVIIa, fXIa, fIXa, fXa and thrombin), some accelerating co-factors (fVa and fVIIIa) and the clottable molecule itself (fibrin) are all formed during the clotting process. fXa and thrombin are the last two factors among the proteases formed. Thrombin, formed upon the action of fXa, initiates the fission of fibrinogen, resulting in the fibrin clot.

According to the earlier concept of blood clotting mechanism [R. G. MacFarlane, Nature **202**, 498 (1964); E. W. Davie and O. D. Ratnoff, Science **145**, 1310 (1964)] fX is activated in two ways by an intrinsic and an extrinsic pathway. In the former case the process is initiated by the surface-activated fXII (fXIIa) with the transformation fXI→fXIa which is followed by the reaction fIX→fIXa; *fX is activated by fIXa.* In the extrinsic pathway the process is initiated by the appearance of the cellular surface receptor, the tissue factor (TF), and the development of the [fVIIxTF] or [fVIIaxTF] complex. *fX is activated by the [fVIIaxTF] complex.*

According to recent findings blood clotting in the living organism is a result of both pathways combined [E. W. Davie et al., Biochemistry **43**, 10363 (1991)] where the main steps are the following:

1. In the case of vessel wall injury or disease TF migrates to the surface and binds a portion of factor VII circulating in the blood. The [fVII.TF]-complex formed is converted by the action of suitable trace amounts of proteases (e. g. fXIIa, fXa, fIXa and thrombin) into the active enzyme complex [fVIIa.TF] which activates a small portion of plasma factors IX and X (i. e. small amounts of fIXa and fXa are formed), then it is inactivated by the action of TFPI (Tissue Factor Pathway Inhibitor; earlier name LACI Lipo-protein-Associated Coagulation Inhibitor), the common inhibitor of both fXa and [fVIIaxTF]-complex [T. J. Girard et al., Nature **338**, 518-520 (1989)].

2. The generated fIXa, together with factor X and cofactor VIIIa, produces, in the presence of Ca^{++} ions, on a phospholipid surface (PL) the "tenase" complex, [fIXa.fVIIIa.fX.PL.Ca^{++}], wherein fX is activated to fXa.

3. The fXa generated up to this point, together with prothrombin (fII) and cofactor fVa, produces the "prothrombinase complex" [fXa.fVa.fII.PL.Ca^{++}], which has a similar structure as "tenase". Here prothrombin is converted to thrombin. The fV→fVa and fVIII→fVIIIa conversions are performed either by fXa or thrombin.

4. The small amount of thrombin generated converts a portion of fXI to the enzyme fXIa and activates parts of factors VIII and V, to produce further amounts of fVIIIa and

fVa, resp. By now fXIa can carry out the conversion of factor IX to the enzyme fIXa. With this step the chain reaction starting with the Xase-complex and terminated with thrombin formation is resumed. With the repetition of the process increasing amounts of thrombin are formed.

5. At a suitable high thrombin concentration the fibrinogen dissolved in the plasma undergoes partial proteolysis, a fibrin-monomer is generated which is first associated to the soluble fibrin polymer, then it is converted to insoluble fibrin polymer. Here also the thrombin is playing a role as fXIIIa, the factor performing polymerization, is produced upon its action [L. Lorand and K. Konishi, Arch. Biochem. Biophys. **105**, 58 (1964)].

The insoluble fibrin polymer is the main component of the blood clot and thrombus, the other component is the blood platelet aggregate which is generated primarily upon the action of thrombin, too. The thrombus or blood clot formed entraps the major part of thrombin generated during the process which triggers a new coagulation process when it gets into the solution during the dissolution of the thrombus [A. K. Gash et al., Am. J. Cardiol. **57**, 175 (1986); R. Kumar et al., Thromb. Haemost. **72**, 713 (1994)].

The above features demonstrate the key role of thrombin in thrombus formation. Consequently, all compounds interfering with the function and/or formation of thrombin are of major importance in the therapy of thrombosis and related diseases.

At present the most widely and successfully used compounds applied for the prophylaxis and treatment of

thrombosis are the heparins and the vitamin K antagonist coumarins (e. g. Syncumar and Warfarin) which are indirect thrombin inhibitors.

Heparin catalyses the reaction beween thrombin and its natural inhibitor, antithrombin-III (AT-III). However, this action of heparin is absent if the plasma concentration of AT-III is lower than 75 % of the normal level [R. Egbring et al., Thromb. Haemost. **42**, 225 (1979)]. It is also of importance that the thrombin bound by the above-mentioned thrombus fails to be inhibited by this indirect mechanism as it is inaccessible to the heparin-AT-III-complex [J. I. Weitz et al., J. Clin. Invest. **86**, 385 (1990)]. In addition, side effects such as treatment related haemorrhages and thrombo-embolies developing due to immunopathological processes are not negligible either [J. M. Walenga et al., Clin. Appl. Thrombosis/Hemostasis, **2(Suppl.1)**, S21-S27 (1996)].

The vitamin K antagonists may be administered also orally, their effect is developing after 16-24 hours. They inhibit the development of the reactive forms of some clotting factors with Gla content (i. e. prothrombin). To achieve therapeutic effects partial inhibition (60-70 %) is required [M. P. Esnouf and C. V. Prowse, Biochim. Biophys. Acta **490**, 471 (1977)] which may be attained by suitable drug dosage. The use of vitamin K antagonists, however, is not easy due to their narrow therapeutic range, strong dependence on diet composition (vitamin K) and variable individual sensitivity.

The first highly potent synthetic compound directly inhibiting thrombin was the tripeptide aldehyde D-Phe-Pro-Arg-H, a reversible inhibitor, exhibiting significant anticoagulant activity both *in vitro* and *in vivo* [S. Bajusz et al., in: Peptides: Chemistry, Structure and Biology (R. Walter and J. Meienhofer, Eds.), Ann Arbor Publ., Ann Arbor, Michigan, USA, 603-608 (1975); Int. J. Peptide Protein Res. **12**, 217 (1978)]. A series of compounds related to D-Phe-Pro-Arg-H have been synthesized. One of the first was Boc-D-Phe-Pro-Arg-H [S. Bajusz et al., Int. J. Peptide Protein Res. **12**, 217 (1978)] and the chloromethylketone analogue (D-Phe-Pro-Arg-CH_2Cl) which proved to be an irreversible inhibitor [C. Kettner and E. Shaw, Thromb. Res. **14**, 969 (1979)]. Further peptides and acylpeptides to be mentioned are the boroarginine analogues (D-Phe- and Boc-D-Phe- as well as Ac-D-Phe-Pro-boroArg) which are potent reversible thrombin inhibitors [C. Kettner et al., J. Biol. Chem. **265**,18289 (1990)] and other analogues of Boc-D-Phe-Pro-Arg-H, including the Boc-D-Chg-Pro-Arg-H analogue [(P. D. Geselichen and R. T. Shuman, European patent specification No. 0,479,489 A2 (1992)].

In aqueous solutions D-Phe-Pro-Arg-H was prone to undergo spontaneous conversion, but D-MePhe-Pro-Arg-H (GYKI-14766), obtained by methylating the terminal amino group, already proved to be of suitable stability while retaining the activity of the parent compound [S. Bajusz et al., U. S. patent specification No. 4,703,036 (1987); J. Med. Chem. **33**, 1729 (1990)]. Blood clotting and thrombus formation were significantly inhibited in laboratory animals

by the compound [D. Bagdy et al., Thromb. Haemost. **67**, 357 and **68**, 125 (1992); J. V. Jackson et al., J. Pharm. Exp. Ther. **261**, 546 (1992)]; its inhibitory action on the enzymes of fibrinolysis was negligible, co-administered with thrombolytics it significantly promoted the dissolution of the thrombus [C. V. Jackson et al., J. Cardiovascular Pharmacol. **21**, 587 (1993)] which could not be attained with the heparin AT-III complex. Several compounds related to D-MePhe-Pro--Arg-H have been synthesized, e. g. D-MePhg-Pro-Arg-H [R. T. Shuman et al., J. Med. Chem. **36**, 314 (1993)].

New, stable and potent analogues of D-Phe-Pro-Arg-H have been obtained by substituting an α-hydroxyacyl group for the terminal Phe-moiety. Some of them, e. g. the D-2-cyclohexyl-2-hydroxyacetyl analogue, D-Hma-Pro-Arg-H, exhibited, similarly to **C1**, very high anticoagulant and anti-thrombotic effect [Hungarian patent specification No. 211,088; S. Bajusz et al., Bioorg. Med. Chem. **8**, 1079 (1995)].

Anticoagulant activity (i. e. inhibition of the proteolytic reactions in the process) is measured by *anticoagulant tests*, e. g. by the thrombin time (*TT*), activated partial thromboplastin time (*APTT*) and prothrombin time (*PT*) tests [E. J. W. Bovie et al; Mayo Clinic Laboratory Manual of Hemostasis; W. B. Sanuders Co., Philadelphia (1971)]. Plasma, inhibited in spontaneous coagulation, e. g. citrate-plasma, is made to coagulate and the required coagulation time is measured. Upon the action of anticoagulants the coagulation time is prolonged proportionally to the inhibition of the reaction(s) in the process. The anticoagulant effect

can be characterized by the substance concentration required to double the coagulation time compared to the control (IC_{50}). The effect of anticoagulants on individual coagulant proteases is measured by the *amidolytic method* [R. Lottenberg et al., Methods in Enzymol. **80**, 341 (1981); G. Cleason, Blood Coagulation and Fibrinolysis **5**, 411 (1994)]. The isolated active factor (e. g. thrombin, fXa) and its chromogen or fluorogen peptide-amide substrate are reacted in the presence or absence of the inhibitor, resp. The enzyme inhibiting action is characterized by the inhibitory constant (IC_{50}) measured during amidolysis.

In the TT test coagulation is initiated by the thrombin added to the citrate plasma. In the system 22 pmol/mL of thrombin is functioning and in the presence of plasma components its inhibition can be measured on the fibrinogen (one of the natural substrates of thrombin) present. In the APTT and PT tests the full coagulation process is made to proceed. Depending on the activator fX is activated by the extrinsic or intrinsic pathway. The generated fXa activates prothrombin to thrombin which, in turn, triggers plasma coagulation. In the test the coagulation time is prolonged if the enzymes or one of them is inhibited by the inhibitor. In the APTT and PT tests at most 40 pmole/ml Xa is generated (this is the full amount of factor X present in both systems) while 150 pmole/ml (APTT) and 350 pmole/ml (PT) are generated from thrombin [B. Kaiser et al., Thromb. Res. **65**, 157 (1992)].

In the case of D-MePhe-Pro-Arg-H (**C1**) the concentration doubling the clotting time in the TT, APTT and

PT tests amounted to 87, 622 and 2915 nM, resp. These values and the amount of thrombin (22, 150 and 350 pmole/ml) functioning in the tests increased similarly, suggesting that **C1** behaves as a thrombin inhibitor in both the APTT and the PT tests and had only slight or no influence on the function of fXa. In good agreement with these results the amidolytic effect of thrombin on the Tos-Gly-Pro-Arg-pNA substrate was inhibited by **C1** with an IC_{50} = 2 nM value, while the amidolytic effect of fXa on the corresponding Bz-Ile-Glu-Gly-Arg-pNA substrate was only slightly affected with an IC_{50} = 9,1 mM value (Bajusz et al. unpublished results).

It is an inherent characteristic of the blood clotting mechanism that the process is inhibited not only by direct thrombin inhibitors but also by factors blocking thrombin formation, e. g. fXa inhibitors. The *60-member polypeptide isolated from tick*, TAP (Tick Anticoagulant Peptide) [L. Waksman et al., Science **248**, 593 (1990); A. B. Kelly et al., Circulation **86**, 1411 (1992)] and DX-9065a (**C2**), a *synthetic non-peptide*, (+)-(2S)-2-[4[[(3S)-1-acetimidoyl-3--pyrrolidinyl]oxy]phenyl]-3-[7[amidino-2-naphthyl]-propionic acid hydrochloride pentahydrate [T. Hara et al., Throm. Haemost. **71**, 314 (1994); T. Yokoyama et al., Circulation **92**, 485 (1995)] are strong inhibitors of blood clotting and thrombus formation. Both the amidolytic effect and plasma clotting of fXa are strongly inhibited in the PT and APTT tests by these compounds, i. e. both the free and the complex-bound Xa factors are equally well inhibited while according to their nature as specific fXa inhibitors thrombin

is not inhibited at all, i. e. they fail to exert any activity in the TT test. 4-MeP-Asp-Pro-Arg-H (**C3**) (international patent application No. 93/15756) and Boc-D-Phe-Nal(1)-Arg-H (C4) (international patent application No. 95/13693) are *synthetic peptide* inhibitors of fXa. According to the literature **C3** and **C4** inhibit the amidolytic activity of fXa on the Z-D-Arg-Gly--Arg-pNA substrate at IC_{50} = 57 and 30 nM, resp. No data are available on their anticoagulant potency. In our own tests **C3** and **C4** exhibited significant inhibition also on the Bz-Ile-Glu-Gly-Arg-pNA substrate while their anticoagulant activity proved to be negligible in the plasma clotting tests. The published (**C2**) and measured (**C3-C4**) activities of synthetic fXa inhibitors compared to the antithrombin compound **C1** are presented in Table 1.

The data of Table 1 demonstrate that in the case of **C1** the anticoagulant effect is due to the inhibition of thrombin while in the case of **C2** it is due to the inhibition of fXa. The significant fXa inhibitory effect of **C3** and **C4** is not accompanied by any significant anticoagulant effect. Most probably the fXa active center in the prothrombinase complex is inaccessible to **C3** and **C4,** these peptides can inhibit only free fXa in solution.

Table 1

fXa inhibiting (A) and anticoagulant (B) effect of known synthetic inhibitors

Inhibitor	A: IC_{50}, nM[b]	B: IC_{50}, µM[a]		
		PT	APTT	TT
C1	9133	2.91	0.62	0.09
C2	70	0.52	0.97	NA[c]
C3	64	19.32	4.59	0.87
C4	86	53.62	9.96	17.24

[a]Peptide concentration doubling clotting time compared to the control in the prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) test

[b]Value measured with fXa on Bz-Ile-Glu-Gly-Arg-pNA chromogen substrate

[c]NA = inactive

According to a recent publication [N. A. Pager et al., Circulation **92**, 962 (1995)] not only thrombin but also factor Xa, entrapped in the thrombus/blood clot and liberated during dissolution, contributes to the initiation and maintenance of a new coagulation process through the activation of the [fVII.TF]-complex or factors V and VIII, resp. Consequently, it is advantageous if the anticoagulants are able to inhibit factor Xa in addition to the inhibition of thrombin, particularly if this inhibition is extended to thrombin and factor Xa entrapped in the clot.

Short description of the invention

It is the objective of the present invention to prepare new peptide derivatives with improved anticoagulant activity compared to known compounds which exhibit anticoagulant activity also at oral administration.

It was observed that the amidolytic effect of factor Xa was inhibited at about 30 times lower IC_{50} values by the known α-D-hydroxyacyl-L-prolyl-L-arginine aldehydes [S. Bajusz et al., Bioorg. Med. Chem. **8**, 1079 (1995)] compared to **C1** while they possessed similar significant anticoagulant activity. D-Hma-Pro-Arg-H (**C5**) is such a D-2-cyclohexyl-2-hydroxyacetyl analogue. It was unexpectedly found that the analogues obtained with 2-cycloheptyl-2-hydroxyacetic acid and 2-cyclopentyl-2--hydroxyacetic acid, D-cHga- and D-cPga-Pro-Arg-H, inhibit factor Xa even stronger while retaining significant anticoagulant effect, too.

Detailed description of the invention

This invention relates to new peptide aldehyde derivatives of general formula (I),

D-Xaa-Pro-Arg-H (I)

wherein

Xaa represents a 2-cycloheptyl-2-hydroxyacetyl or 2-cyclopentyl-2-hydroxyacetyl group,

Pro represents an L-prolyl residue and

Arg represents an L-arginyl residue,

their acid-addition salts formed with an organic or inorganic acid and pharmaceutical compositions containing the same.

The compounds of general formula (I), wherein Xaa, Pro and Arg have the same meaning as above, are prepared by condensing an acyldipeptide Q-D-Xaa-Pro, protected on the hydroxy group with a Q protecting group, with an L-arginine lactam, protected on the guanidino group with a benzyl-

oxycarbonyl group, and reducing the obtained protected peptide lactam to the protected peptide aldehyde of the formula Q-D-Xaa-Pro-Arg(Z)-H, finally removing the Z group from the guanidino group of arginine and the Q group from the α-hydroxy group, and isolating the peptide derivative of general formula (I) as its addition salt formed with an organic or inorganic acid.

The acyldipeptide Q-D-Xaa-Pro, used as starting material, is prepared by converting a D-Xaa α-hydroxyacid to a benzyl ester, protecting the hydroxy group with a Q group, converting the resulting Q-D-Xaa-OBzl by hydrogenolysis to the free acid, and coupling it to L-proline benzyl ester. The required O-protected acyl-dipeptide is obtained by removing the benzyl ester with hydrogenolysis.

Q-D-Xaa, the α-hydroxyacid protected by a Q group on the OH group and required for the coupling with proline, is advantageously prepared by O-acetylating the racemic DL-Xaa compound, then removing the acetyl group from Ac--D-Xaa by enzymatic hydrolysis. In a preferred process the racemic DL-Xaa can be resolved with D-tyrosine hydrazide, too.

Compounds of general formula (I) of the invention wherein Xaa, Pro and Arg have the same meaning as above, exhibit strong anticoagulant activity both *in vitro* and *in vivo* and possess excellent bioavailability.

The *in vitro* anticoagulant effect of the compounds of general formula (I) was measured by the prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) tests [D. Bagdy et al., Thromb. Haemost.

67, 325 (1992)]. The Xa-factor-inhibiting effect of the compounds was also determined by using the Bz-Ile-Glu-Gly-

-Arg-pNA chromogen substrate (see method M6).

The results obtained are presented in Table 2. The corresponding data of **C1** and **C5** served as controls. In the Table the compounds are listed in the decreasing order of PT activity. The data definitely demonstrate the beneficial effect of the terminal cHga- and cPga-moieties compared to the Hma and MePhe residues.

Table 2

Anticoagulant (**A**) and factor Xa inhibiting (**B**) effect of the new peptidyl aldehydes of the invention and of control compounds having similar structure in the decreasing order of PT activity

Peptidyl-arginine aldehyde (No.[b])	A: IC_{50}, μM[a]			B: IC_{50}
	PT	APTT	TT	nM[d]
D-cHga-Pro-Arg-H (**1**)	1.20	0.37	0.11	63
D-cPga-Pro-Arg-H (**2**)	2.03	0.71	0.10	107
Control compounds				
D-Hma-Pro-Arg-H (**C5**)	2.14	0.79	0.22	247
D-MePhe-Pro-Arg-H (**C1**)	2.92	0.62	0.09	9133

[a]Peptide concentration doubling thrombin time compared to control
[b]Identical to example number describing preparation of the respective compound and control (**C1**, **C5**)
[d]Value measured on substrate Bz-Ile-Glu-Gly-Arg-pNA with isolated human factor Xa (see methods M1 - M5).

Table 3 demonstrates on compound No. 1 as an example the inhibitory effect of the compounds of general formula (I) on factor Xa, entrapped in plasma clot, and thrombin as well as on thrombin bound to fibrin gel; the corresponding data of **C1** and **C2** served as controls. The plasma clot was

obtained by the recalcification of platelet-rich human citrate plasma and fibrin gel by the coagulation of human fibrinogen with human thrombin. Z-D-Arg-Gly-Arg--pNA and Tos-Gly-Pro-Arg-pNA were used as substrates for measuring the activity of factor Xa and thrombin (methods M1-M5).

Table 3

Inhibitory effect of the new peptidyl-arginine aldehyd (1) of the invention and control compounds (C1, C5) (IC_{50},μM) on plasma clot entrapped factor Xa and thrombin as well as on fibrin-gel bound thrombin[a]

Peptidyl-arginine aldehyde (No.[b])	Plasma-clot		Fibrin-gel
	Factor Xa	Thrombin	Thrombin
D-cHga-Pro-Arg-H (1)	0.20	0.52	0.21
Control compounds			
D-MePhe-Pro-Arg-H (C1)	1.12	0.38	0.30
D-Hma-Pro-Arg-H (C5)	0.19	0.27	0.22

[a] Moc-D-Chg-Gly-Arg-pNA and Tos-Gly-Pro-Arg-pNA served as substrates for measuring the activity of factor Xa and thrombin at the determination of IC_{50} values according to methods M1-M5

[b] Identical to example number describing preparation of the new compound and controls (C1, C5)

The data demonstrate that factor Xa and thrombin, entrapped in platelet-rich human plasma clot as well as thrombin bound to fibrin-gel are inhibited by compound (1) of the invention at IC_{50} values lower than micromole/nanomol, similarly to compound C5.

The inhibitory effect of the new compounds of the invention on plasmin (PL) as well as on plasmin formation induced by tissue plasminogen activator (tPA) and urokinase (UK) was studied by the fibrin-platelet method [D. Bagdy et al.: Thromb. Haemost. 67, 325 (1992)].

Compounds **C1** and **C3** served as controls. The moderate antifibrinolytic action of **C1** proved to be negligible *in vivo* and it could be used as an adjuvant at the dissolution of experimental thrombus while the antifibrinolytic activity of **C3** was detectable *in vivo* [C. V. Jackson et al., J. Cardiovasc. Pharmacol. **21**, 587 (1993)].

In Table 4 the results obtained with the new compounds **1** and **2** as well as with controls **C1** and **C3** are presented as an example. In addition to the IC_{50} values (columns A) the efficacy of the compounds related to **C1** are also listed (columns B). The latter data indicate that similarly to compound **C1** the antifibrinolytic activity of the new compounds **1** and **2** is moderate, their activity against the 3 enzymes tested is 7.5 - 12-39 times lower than that of compound **C3**.

Table 4

The inhibitory effect (IC_{50}) of the new peptidyl-arginine aldehydes of the invention and of controls with similar structure on plasmin (PL) as well as on plasmin formation induced by tissue plasminogen activator (tPA) and urokinase (UK) studied by the fibrin-plate method[a]

Peptidyl-arginine aldehyde (No.[c])	A: IC_{50} and B: relative efficacy[b]					
	PL		tPA		UK	
	A	B	A	B	A	B
D-cHga-Pro-Arg-H (**1**)	83	0.6	74	1.8	120	0.7
D-cPga-Pro-Arg-H (**2**)	3.9	0.13	112	1.2	137	0.6
D-MePhe-Pro-Arg-H (**C1**)	54	1.0	132	1.0	82	1.0
Boc-D-Phe-Pro-Arg-H (**C3**)	12	4.5	6	22.0	3	27.3

[a] IC_{50} = peptide concentration (mM) where the hyrolyzed area on the fibrin plate is reduced by 50 % compared to the control

[b] Values related to the activity of **C1** ($1/IC_{50} = 1$)

[c] Identical to example number describing preparation of the respective compound and control (**C1**, **C3**)

The anticoagulant and platelet aggregation inhibiting effect of the compounds of general formula (I) was studied in New Zealand white rabbits *ex vivo* according to D. Bagdy et al. [Thromb. Haemost. **67**, 357 (1992). The compounds were dissolved in buffered isotonic saline solution and administered i. v. (0,04-5.0 mg/kg) or by infusion (0.25-5.0 mg/kg/h) or subcutaneously (0.5-6.0 mg/kg) or p.o. (2.5-20 mg/kg). The effect of the compounds was detectable already within 30 minutes following p.o. administration, peak values were attained after 60-180 minutes and the therapeutic level was maintained dose-dependently for 3->6 hours.

The *in vivo* effect of D-cHga-Pro-Arg-H (1) is presented in detail in Tables 5 and 6. The corresponding values of **C1** are listed as controls. The compound was administered p.o. in doses of 5 mg/kg. The blood samples drawn from the caudal vein in every 30-60 minutes were analyzed. The whole blood clotting time (WBCT) and the inhibition of thrombin-induced blood platelet aggregation (PAI) were determined. The activated partial thromboplastin time (APTT) and the thrombin time (TT) in the citrate plasma obtained from the blood sample were also measured. The APTT and TT values are compiled in Table 5 and the WBCT and PAI values in Table 6.

Table 5

Anticoagulant effect of D-cHga-Pro-Arg-H and D-MePhe-Pro-Arg-H in rabbits at p.o. doses of 5 mg/kg in the APTT and TT tests characterized by relative clotting times[a]

Time	D-cHga-Pro-Arg-H (1)		D-MePhe-Pro-Arg-H (C1)	
min.	APTT	TT	APTT	TT
0	1.0	1.0	1.0	1.0
30	*2.01±0.43*	*10.57±5.98*	1.27±0.38	1.52±0.17
45	*2.25±0.43*	*10.89±5.89*	1.30±0.02	2.50±0.44
60	*2.32±0.43*	*11.67±5.69*	1.37±0.03	*4.75±1.38*
90	*2.11±0.31*	*14.00±6.48*	1.45±0.09	*10.50±5.49*
120	*1.84±0.11*	*10.60±4.22*	1.43±0.11	5.51±3.59
180	*1.66±0.10*	2.22±0.71	1.39±0.09	2.05±0.38
240	1.37±0.08	1.23±0.07	1.31±0.11	1.81±0.39
300	1.31±0.09	1.13±0.05	1.25±0.22	-

[a]Ratio of clotting times measured in treated and untreated animals. *Therapeutic values are in italics.*

Table 6

Anticoagulant and blood platelet aggregation inhibiting (PAI) effect of D-cHga-Pro-Arg-H and D-MePhe-Pro-Arg-H in rabbits at p.o. doses of 5 mg/kg in the WBCT test characterized by relative clotting time and percentual inhibition[a]

Time	D-cHga-Pro-Arg-H (1)		D-MePhe-Pro-Arg-H (C1)	
min.	WBCT	PAI(%)	WBCT	PAI(%)
0	1.0	0	1.0	0
30	*1.80±0.36*	*71.0±19.4*	1.07±0.13	*52.8±14.7*
45	*2.03±0.35*	*74.0±13.9*	1.26±0.07	*58.4±15.6*
60	*1.98±0.30*	*91.0±4.5*	*1.55±0.17*	*54.2±11.6*
90	*1.78±0.26*	*93.0±3.5*	*1.61±0.34*	*83.2±8.9*
120	*1.52±0.13*	*92.4±6.5*	1.45±0.21	*75.2±12.3*
180	1.29±0.07	71.6±19.8	1.44±0.08	47.5±20.9
240	1.13±0.04	49.4±21.5	1.22±0.08	32.2±16.0

[a]Ratio of clotting times measured in treated and untreated animals. *Therapeutic values are in italics.*

The data in the Tables show that the new compound 1 has higher and more stable anticoagulant and blood platelet

aggregation inhibiting activity than the control compound **C1**.

The compounds of the invention of general formula (I) are used for the treatment and prevention of the following diseases where thrombosis and/or hypercoagulability is involved: deep venous thrombosis, pulmonary embolism, arterial thrombosis, unstable angina, myocardiac infarct, auricular fibrillation and thrombosis-based stroke. In atherosclerosis they may be used to prevent diseases of coronary arteries, thrombotic diseases of cerebral arteries, as a surgical prophylaxis of high risk patients or other surgical prophylaxis. They may be applied in the thrombolysis of percutaneous transluminal angioplastics for the prevention of reocclusion, for adjuvant therapy in nephrosis in the case of hemodialysis and in diseases with hypercoagulability: in malignant tumours and inflammation (e. g. arthritis) as well as in diabetes. They may be applied in cases where the administration of other anticoagulants fails to be effective or is contraindicated, e.g. lack of antithrombin-III in the case of heparin or heparin-induced thrombocytopenia (HIT), and in the case of coumarins, e. g. pregnancy.

The compounds of the invention and their pharmaceutically acceptable salts are used for therapeutic purposes alone or preferably in the form of a pharmaceutical formulation. The invention also refers to these formulations.

The pharmaceutical formulations comprise an effective amount of a compound of general formula (I) or a pharmaceutically acceptable salt thereof and known

pharmaceutically acceptable carriers, filling materials, diluents and/or other pharmaceutical excipients.

The above carriers, diluents or filling materials can be water, alcohols, gelatin, lactose, saccharose, starch, pectin, magnesium stearate, stearic acid, talcum, various oils of animal or plant origin, furthermore glycols, e. g. propylene glycol or polyethylene glycol. The pharmaceutical excipients can be preservatives, various natural or synthetic emulgeators, dispersing or wetting agents, colouring materials, flavouring agents, buffers, materials promoting disintegration and other materials improving the bioavailability of the active ingredient.

The pharmaceutical compositions of the invention can be prepared in usual formulations such as oral compositions (administered through the mouth such as tablets, capsules, powders, pills, dragées or granulates) as well as parenteral compositions (drugs administered by avoiding the gastrointestinal system such as injections, infusions, suppositories, plasters or ointments).

The therapeutic dose level of the compounds of the invention depends on the individual health status and age of the patient and may vary accordingly; consequently, its level is fixed by the physician designing treatment. In diseases where inhibiton of the function and/or formation of thrombin is required for prophylactic or therapeutic purposes a daily oral or parenteral (e. g. i. v.) dose of 0.01 to 1000 mg/kg body weight, preferably 0.25 to 20 mg/kg body weight, may be administered.

The compounds of general formula (I) of the invention, administered together with thrombolytic agents (e. g. tPA or urokinase), actively promote the dissolution of thrombi formed in arteries or veins and efficiently prevent their reformation. In such cases it is preferred to administer the compounds of the invention simultaneously with thrombolytic agents or immediately after thrombolytic treatment.

The following examples are illustrating but not limiting the scope of the invention.

The R_f values recorded in the examples were determined by thin-layer chromatography, using silica gel as adsorbent (DC-Alufolien Kieselgel 60 F_{254}, Merck, Darmstadt), in the following developing solvents:

1. Ethyl acetate
2. Ethyl acetate - pyridine - acetic acid - water (480:20:6:11)
3. Ethyl acetate - pyridine - acetic acid - water (60:20:6:11)
6. Ethyl acetate - pyridine - acetic acid - water (240:20:6:11)
12. Ethyl acetate - cyclohexane (1:9)
14. Chloroform - acetic acid (95:5)

The capacity factors (k') specified in the examples were determined with the apparatus "Pharmacia LKB Analytical HPLC System Two" as follows:

Column: "VYDAC C-18 reversed phase: 10 µm, 300 A, 50 mm"

Buffer A: 0.1 % trifluoroacetic acid in water

Buffer B: 0.1 % trifluoroacetic acid in acetonitrile

Gradients applied (flow rate) at 1 mL/min.

I: 0-5 min. 0-25 % B, then isocratic 25 % B;

II: 0-30 min. 0-60 % B.

The gradient applied in the HPLC analysis (I or II) is specified in brackets after the abbreviation at the individual steps of the examples.

Optical purity was determined with the above HPLC apparatus as follows and indicated with the expression "chiral HPLC" in brackets:

Column: Chiralpack WH (DAICEL) 4x250 mm

Eluant: 0.25 mM $CuSO_4$; flow rate 1 mL/min.

Analyses were performed at 50°C.

The peptide content of the eluate was detected in UV light at 214 nm. Sample concentration: 1 mg/mL in buffer A (reverse phase) or methanol (chiral phase) resp.; injected volume 25 µL.

The acylarginine aldehydes are present in equilibrium structures, i. e. in aldehyde, aldehyde hydrate and two aminocyclol forms. During HPLC analysis the aldehyde hydrate and one or both aminocyclol forms appear as separate peaks, consequently the acylarginine aldehydes described in the examples are specified by two or three k' values.

Mass spectrometry. The FAB positive ionization measurements were performed in a Finnigan MAT 8430 apparatus. The samples were dissolved in m-nitrobenzyl-alcohol matrix and introduced directly into the ion source. In

the spectrum of peptidyl-arginine aldehydes an additional molecule ion was detectable, that of the addition compound formed with m-nitrobenzylalcohol (NBA): $[M+H]^+$ and $[M+H+NBA]^+$. In the examples the FAB spectral data were specified accordingly. The ESI positive ionization measurements were performed in a VG Quattro (Fisons) apparatus. The samples were dissolved in a mixture of acetonitrile - water (1:1) containing 1 % (v/v) of formic acid and were introduced with a 10 mL sample-loop into the ion source at a flow rate of 15 - 25 mL/min.

The specific rotations ($[\alpha]_D$) were determined at 20°C.

Example 1

D-2-Cycloheptyl-2-hydroxyacetyl-L-prolyl-L-arginine aldehyde (D-cHga-Pro-Arg-H) hemisulfate

Step 1: Tetrahydropyranyl-D-2-cycloheptyl-2--hydroxyacetyl-L-prolyl-N^G-benzyloxycarbonyl-L--arginine lactam

7.85 g (20.1 mmole) of tert-butyloxycarbonyl-N^G--benzyloxycarbonyl-L-arginine lactam [(S. Bajusz et al., J.Med.Chem. **33**, 1729 (1990)] is suspended in 20 mL of chloroform, then 20 mL of ethyl acetate saturated with HCl gas (0.11 - 0.15 g/mL) is added under stirring and ice-cooling. The cleaving of the Boc group is monitored by thin--layer chromatography [$R_f(3)$ = 0.5 (free compound); 1.0 (Boc-compound)]. By the end of the reaction the suspension is diluted with 40 mL of diethyl ether, the crystal mass formed is filtered, washed with 10 mL of acetone and 10 mL of diethyl ether, and dried at reduced pressure over

potassium hydroxide for two hours. The resulting N^G-benzyloxy-carbonyl-L-arginine lactam hydrochloride is dissolved in 20 mL of dimethyl formamide, cooled to -20°C and added to the following mixed anhydride.

7.1 g (20.1 mmole) of tetrahydropyranyl-D-2-cyclo-heptyl-2-hydroxyacetyl-L-proline (Example 1, Step 1) is dissolved in 20 mL of dimethyl formamide, cooled to -20°C, then under stirring 2.23 mL (20.1 mmole) of N-methyl-morpholine, 2.65 mL (20.1 mmole) of isobutyl chloroformate, after 10 minutes of stirring the above dimethyl formamide solution of N^G-benzyloxycarbonyl-L-arginine lactam and finally triethylamine are added in a quantity to adjust the pH of the reaction mixture to 8 (about 2.8 mL is required). The reaction mixture is stirred at -10°C for 30 minutes, then at 0°C for one hour. Thereafter the salts are filtered off and the filtrate is diluted with 100 mL of ethyl acetate. The resulting solution is washed with 3 x 25 mL of water, 10 mL of 1 M potassium hydrogen sulfate and 3 x 10 mL of water, dried over anhydrous sodium sulfate solution and evaporated at 2.0 - 2.5 kPa. The product obtained is submitted to silica gel column chromatography using 200 g of Kieselgel 60 as adsorbent and ethyl acetate as eluent. The fractions containing solely the pure product [($R_f(1)$ = 0.60] are pooled and evaporated at 2.0 - 2.5 kPa. The evaporation residue is recrystallized from diisopropyl ether.

Yield 8.1 g (64 %), $R_f(1)$ = 0.6

M.p.: 66 - 68°C.

FAB mass spectrum (626 $[M+H]^+$, 779 $[M+H+NBA]^+$) confirms the assumed structure.

Step 2: Tetrahydropyranyl-D-2-cycloheptyl-2-hydroxy--acetyl-L-prolyl-N^G-benzyloxycarbonyl-L-arginine aldehyde

8.0 g (12.8 mmole) of tetrahydropyranyl-D-2-cycloheptyl-2-hydroxyacetyl-L-prolyl-N^G-benzyloxycarbonyl-L--arginine lactam (Example 1, Step 1) is dissolved in 15 ml of tetrahydrofuran, then under stirring and at temperatures not exceeding -50°C a solution of 3.6 mmole of lithium aluminium hydride dissolved in tetrahydrofuran is added. The progress of reduction is monitored by thin-layer chromatography using ethyl acetate - pyridine - acetic acid - water (240:20:6:11) as developing solvent and, if required, a further portion of lithium aluminium hydride is added. To this reaction mixture 0.5 M of sulfuric acid is added dropwise under constant stirring and cooling until pH 3 is attained, then 35 mL of water is added. The resulting solution is extracted with 2 x 15 mL of hexane, then with 3 x 20 mL of methylene chloride. The methylene chloride extracts are pooled, washed with 3 x 15 mL of water, 15 mL of cold 5 % sodium hydrogen carbonate solution and again with 15 mL of water, dried over anhydrous sodium sulfate and evaporated at 2.0 - 2.5 kPa. The evaporation residue is treated with diisopropyl ether, filtered and dried at reduced pressure.

Yield 7.25 g (90 %), $R_f(6) = 0.40$

M.p.: 107°C

$[\alpha]_D = +16.0°$ (c = 1, tetrahydrofuran)

FAB mass spectrum (628 $[M+H]^+$, 731 $[M+H+NBA]^+$)

confirms the assumed structure.

Step 3: D-2-Cycloheptyl-2-hydroxyacetyl-L-prolyl-L--arginine aldehyde hemisulfate

7.05 g (11.23 mmole) of tetrahydropyranyl-D-2--cycloheptyl-2-hydroxyacetyl-L-prolyl-N^G-benzyloxycarbonyl--L-arginine aldehyde (Example 1, Step 2) is dissolved in 60 mL of ethanol, then 11.23 mL of 0.5 M of sulfuric acid, 13.3 mL of water and 0.7 g Pd-C catalyst, suspended in 25 mL ethanol, are added and the mixture is hydrogenated at about 10°C. The progress of the reaction is monitored by thin-layer chromatography and is completed in about 15 minutes. The catalyst is filtered and the filtrate is concentrated to about 7 - 9 mL at 2.0 - 2.5 kPa. The residue is diluted with 80 mL of water, extracted with 4 x 15 mL of methylene chloride and the aqueous solution is left to stand at 20 - 22°C for 24 hours. The solution is again extracted with 3 x 15 mL of methylene chloride and the pH is adjusted to 3.5 with ion-exchange resin Dowex AG 1-X8 (HO^-), then the solution is freeze-dried.

Yield 4.28 g (83 %)

$[\alpha]_D$ = -94.7° (c = 1, water)

HPLC(I): k' = 4.93 and 5.25.

FAB mass spectrum (410 $[M+H]^+$, 563 $[M+H+NBA]^+$) confirms the assumed structure.

The starting material can be prepared as follows:

Tetrahydropyranyl-D-2-cycloheptyl-2-hydroxyacetyl-L--proline (THP-D-cHga-Pro)

Step A: O-Acetyl-DL-2-cycloheptyl-2-hydroxyacetic acid dicyclohexylammonium salt

1.72 g (10 mmole) of DL-2-cycloheptyl-2-hydroxyacetic acid [H. Takeshita et al., Bull. Chem. Soc. Japan **47**, 1767 (1974)] is dissolved in 20 ml of anhydrous pyridine, 9.4 mL (100 mmole) of acetic anhydride is added and the solution is left to stand at room temperature for 24 hours, then it is evaporated at 2.0 - 2.5 kPa and the evaporation residue is dissolved in 25 mL of diethyl ether. The solution is washed with water, 1 M potassium hydrogen sulfate solution and again with water, then it is dried over anhydrous sodium sulfate and evaporated. The residue is dissolved in diethyl ether, then 2.1 mL (10.5 mmole) of dicyclohexylamine and 20 mL of cyclohexane are added. The solution is left in the refrigerator for 2 days. The crystals formed are filtered, washed with 3 x 10 mL of n-hexane and air-dried.

Yield 2.56 g (64.6 %)

Analysis for $C_{23}H_{41}NO_4$ (395.57)

Calculated: C% = 69.83; H% = 10.45; N% = 3.54;

Found: C% = 69.9; H% = 10.7; N% = 3.5.

Step B: Resolution of O-acetyl-DL-2-cycloheptyl-2--hydroxyacetic acid with acylase I enzyme

1.58 g (4.0 mmole) of O-acetyl-DL-2-cycloheptyl-2--hydroxyacetic acid dicyclohexylammonium salt is dissolved in 15 mL of diethyl ether and 5 mL of 1 M potassium hydrogen sulfate solution. The phases are separated, the diethyl ether phase is washed to neutrality, dried over anhydrous sodium sulfate and evaporated. The residue, 0.86 g (4.0 mmole) of an oil - O-acetyl-DL-2-cycloheptyl-2--hydroxyacetic acid - is dissolved in 20 mL of 0.2 M of sodium hydrogen carbonate, then 0.01 g of cobalt(II)chloride hexahydrate and 0.005 mg of acylase I enzyme (Sigma, 2000 - 3000 units/mg) are added and the solution is left to stand at about 25°C for 3 days. The aqueous solution is acidified with 4.5 mL of 1 M potassium hydrogen sulfate solution and extracted with 3 x 10 mL of diethyl ether. The diethyl ether phases are pooled, washed with water, dried over anhydrous sodium sulfate and evaporated. The evaporation residue is an oil, a mixture of L-2-cyclo-heptyl-2-hydroxyacetic acid [R_f(14) = 0.2], formed upon the action of the enzyme, and O-acetyl-D-2-cycloheptyl-hydroxyacetic acid [R_f(14) = 0.5] which is not cleaved by the enzyme. The mixture is submitted to chromatography on a silica gel column, using a mixture of chloroform - acetic acid (95:5) as developing solvent. The fractions containing solely the pure product are pooled and evaporated at 2.0 - 2.5 kPa.

Evaporation residue 1 is O-acetyl-D-2-cycloheptyl-2--hydroxyacetic acid [R_f(14) = 0.2], 0.34 g (79 %) of an oil which is used directly in the next step C.

Evaporation residue 2 is L-2-cycloheptyl-2-hydroxy-acetic acid [R_f(14) = 0.2], 0.25 g (80 %) of a solid compound, which is filtered with n-hexane and air-dried.

M. p.: 78°C

$[\alpha]_D$ = +18.15° (c = 1, methanol). Optical purity about 95 % (chiral HPLC).

Step C: D-2-Cycloheptyl-2-hydroxyacetic acid (deacetylation of O-acetyl derivative with sodium methylate)

O-Acetyl-D-2-cycloheptyl-2-hydroxyacetic acid (evaporation residue 1), obtained in Step B, is dissolved in 4 mL of methanol containing 1.59 mmole of sodium methylate. The solution is left to stand for 24 hours, then it is evaporated. The residue is dissolved in 10 mL of diethyl ether and 2.5 mL of 0.5 M potassium hydrogen sulfate solution, the diethyl ether phase is washed with water, dried over anhydrous sodium sulfate and evaporated. The crystalline residue is worked up with hexane, filtered and air-dried.

Yield 0.21 g (61 %)

$[\alpha]_D$ = -14.7° (c = 1, methanol). Optical purity about 86 % (chiral HPLC).

Step D: D-2-Cycloheptyl-2-hydroxyacetic acid (resolution of DL-compound with D-tyrosine)

17.2 g (100 mmole) of DL-2-cycloheptyl-2-hydroxyacetic acid and 19.52 g (100 mmole) of D-tyrosine hydrazide are suspended in 600 mL of anhydrous ethanol and refluxed up to full dissolution. The solution is left to cool first to room temperature, then it is left in the refrigerator overnight. The crystals formed are filtered, washed with 2 x 20 mL of anhydrous ethanol and dried under reduced pressure. The product, 19.54 g (53.18 mmole) of diastereomer salt, is recrystallized twice from 15 mL/g of anhydrous ethanol. 12.45 g (67.7 %) of the diastereomer salt is obtained which is dissolved in 50 mL of diethyl ether and 50 mL of 1 M potassium hydrogen sulfate. The diethyl ether phase is washed with water to neutrality, dried over anhydrous sodium sulfate and evaporated at 2.0 - 2.5 kPa. The crystalline residue is worked up with n-hexane, filtered and air-dried.

Yield 5.61 g (32.57 mmole, 65.2 %) of D-2-cycloheptyl-2-hydroxyacetic acid.

M. p.: 80°C

$[\alpha]_D$ = -20.2° (c = 1, methanol) and -30° (c = 1, acetic acid).

Optical purity >98 % (chiral HPLC).

Analysis for $C_9H_{16}O_3$ (172.22)

Calculated: C% = 62.76; H% = 9.36;

Found: C% = 62.01; H% = 9.31.

Step E: D-2-Cycloheptyl-2-hydroxyacetic acid benzyl ester

5.51 g (32 mmole) of D-2-cycloheptyl-2-hydroxyacetic acid (Example 1, Step D) is dissolved in 37 mL of dimethyl formamide, then 3.7 mL (31.3 mmole) of benzyl bromide and 6.34 mL (32 mmole) of dicyclohexyl carbodiimide are added and the mixture is stirred for 24 hours at room temperature. Thereafter the reaction mixture is filtered and evaporated at 2.0 - 2.5 kPa. The residue is dissolved in 50 mL of diethyl ether and 50 mL of 0.5 M potassium hydrogen sulfate. The diethyl ether phase is washed with water to neutrality, 2 x 10 mL of 5 % sodium hydrogen carbonate and 2 x 10 mL of water, dried over anhydrous sodium sulfate and evaporated at 2.0 - 2.5 kPa.

Yield 7.4 g (88 %) oil, $R_f(12) = 0.20$

$[\alpha]_D = +13.2°$ (c = 1, methanol).

Step F: Tetrahydropyranyl-D-2-cycloheptyl-2-hydroxy-acetic acid benzyl ester

7.4 g (28 mmole) of D-2-cycloheptyl-2-hydroxyacetic acid benzyl ester (Example 1, Step E) is dissolved in 65 mL of methylene chloride, then 3.6 mL (39.4 mmole) of 2,4-dihydropyrane and 0.3 mL of ethyl acetate saturated with HCl gas (0.11 - 0.15 g/mL) are added and the solution is left to stand at room temperature for 16 hours. Thereafter the reaction mixture is diluted with 40 mL of methylene chloride,

washed with 3 x 20 mL of water, 3 x 20 mL of cold 5 % sodium hydrogen carbonate solution, again with 2 x 20 mL of water, dried over anhydrous sodium sulfate and evaporated at 2.0 - 2.5 kPa. The residue is considered 28 mmole of tetrahydro-pyranyl-D-2-cycloheptyl-2-hydroxyacetic acid benzyl ester [R_f(12) = 0.30].

Step G: Tetrahydropyranyl-D-2-cycloheptyl-2--hydroxyacetic acid

28 mmole of tetrahydropyranyl-D-2-cycloheptyl-2--hydroxyacetic acid benzyl ester (Example 1, Step F) is dissolved in 50 mL of methanol and hydrogenated in the presence of 0.1 g of Pd-C catalyst. The progress of the reaction is monitored by thin-layer chromatography [R_f(12) = 0.30 (ester); 0.00 (acid); R_f(2) = 1.00 (ester); 0.8 (acid)]. By the end of the reaction the catalyst is filtered, the filtrate is evaporated at 2.0 - 2.5 kPa and the oil residue is dried under reduced pressure.

Yield 6.7 g (25 mmole, 89 %) oil, [R_f(2) = 0.80].

Step H: Tetrahydropyranyl-D-2-cycloheptyl-2--hydroxyacetyl-L-proline benzyl ester

25 mmole of tetrahydropyranyl-D-2-cycloheptyl-2--hydroxyacetic acid (Example 1, Step G) is dissolved in 25 mL of dimethyl formamide, then 2.8 mL (25 mmole) of N-methylmorpholine, 6.04 g (25 mmole) of L-proline benzyl ester hydrochloride, thereafter under stirring and ice-cooling

5.16 g (25 mmole) of dicyclohexyl carbodiimide are added. The reaction mixture is stirred for one hour at 0°C and overnight at room temperature, finally it is filtered and evaporated at 2.0 - 2.5 kPa. The residue is dissolved in 100 mL of diethyl ether, washed with 3 x 20 mL of 5 % sodium hydrogen carbonate solution, water, 1 M potassium hydrogen sulfate solution and water, dried over anhydrous sodium sulfate and evaporated. The evaporation residue is dried under reduced pressure.

Yield 9.9 g (90 %) oil, $R_f(1) = 0.80$.

Step I: Tetrahydropyranyl-D-2-cycloheptyl-2-hydroxy-acetyl-L-proline

9.9 g (22.5 mmole) of tetrahydropyranyl-D-2-cyclo-heptyl-2-hydroxyacetyl-L-proline benzyl ester (Example 1, Step H) is dissolved in 100 mL of methanol and hydrogenated in the presence of 0.1 g of Pd-C catalyst. The progress of the reaction is monitored by thin-layer chromatography [$R_f(1) = 0.80$ (ester); 0.00 (acid); $R_f(2) = 1.00$ (ester); 0.30 (acid)]. By the end of the reaction the catalyst is filtered, the filtrate is evaporated at 2.0 - 2.5 kPa and the oil residue is dried under reduced pressure.

Yield 7.2 g (90 %) oil, $R_f(2) = 0.30$, a major portion of which is directly used in Example 1, Step 1, and a small portion is converted to a crystalline salt as follows.

0.35 g (1 mmole) of tetrahydropyranyl-D-2-cycloheptyl--2-hydroxyacetyl-L-proline (oil) is dissolved in 5 mL of

diethyl ether and 0.115 mL (1.05 mmole) of cyclohexylamine is added. The crystals formed are filtered, washed with diethyl ether and dried at reduced pressure.

Yield 0.40 g (90 %) of tetrahydropyranyl-D-2-cycloheptyl--2-hydroxyacetyl-L-proline cyclohexylammonium salt.

M. p.: 146 -150°C

$[\alpha]_D$ = -12.8° (c = 1, ethanol).

Analysis for $C_{25}H_{44}N_2O_5$ (452.62)

Calculated: C% = 66.34; H% = 9.80; N% = 6.19;

Found: C% = 66.20; H% = 9.90; N% = 6.08.

Example 2

D-2-Cyclopentyl-2-hydroxyacetyl-L-prolyl-L-arginine aldehyde (D-cPga-Pro-Arg-H) hemisulfate

Step 1: Tetrahydropyranyl-D-2-cyclopentyl-2--hydroxyacetyl-L-prolyl-N^G-benzyloxycarbonyl-L--arginine lactam

0.82 g (2.1 mmole) of tert-butyloxy-carbonyl-N^G-benzyloxycarbonyl-L-arginine lactam [S. Bajusz et al., J. Med. Chem. **33**, 1729 (1990)] and tetrahydropyranyl--D-cyclopentyl-2-hydroxyacetyl-L-proline (Example 2, Step G) are coupled. Using the process described in Example 1, Step 1, and utilizing proportional amounts of reagents and solvents, the final product is purified by chromatography. The fractions containing solely the pure final product [R_f (1) = 0,4] are pooled, evaporated at 2.0 - 2.5 kPa and the residue is dried at reduced pressure.

Yield 0.59 g (47 %) oil which is considered 0.97 mmole of

tetrahydropyranyl-D-2-cyclopentyl-2-hydroxyacetyl-L-prolyl--N^G-benzyloxycarbonyl-L-arginine lactam. The FAB mass spectrum confirms the assumed structure: (598 $[M+H]^+$, 751 $[M+H+NBA]^+$.

Step 2: Tetrahydropyranyl-D-2-cyclopentyl-2--hydroxyacetyl-L-prolyl-N^G-benzyloxycarbonyl-L--arginine aldehyde

0.56 g (0.98 mmole) of tetrahydropyranyl-D-2-cyclopentyl-2-hydroxyacetyl-L-prolyl-N^G-benzyloxycarbonyl-L--arginine lactam (Example 2, Step 1) is reduced by the process described in Example 1, Step 2, using proportional amounts of reagents and solvents, except that the thin-layer chromatography monitoring the progress of the reaction is run in ethyl acetate [R_f(1) = 0.4 (lactam), 0.0 (aldehyde)]. Yield 0.4 g (67 %) of the aimed product after working up with hexane. R_f (6) = 0.4 (lactam).

M. p.: 91 - 93°C

The FAB mass spectrum confirms the assumed structure: (600$[M+H]^+$, 753$[M+H+NBA]^+$).

Step 3: D-2-Cyclopentyl-2-hydroxyacetyl-L-prolyl-L--arginine aldehyde hemisulfate

0.38 g (0.63 mmole) of tetrahydropyranyl-D-2-cyclopentyl-2-hydroxyacetyl-L-prolyl-N^G-benzyloxycarbonyl-L--arginine aldehyde (Example 2, Step 2) is transformed, by the process described in Example 1, Step 3, using proportional amounts of reagents and solvents,

Yield 0.22 g (81 %) of the aimed product. HPLC(I):

k' = 4.29 and 4.68.

The FAB mass spectrum confirms the assumed structure:

(382[M+H]$^+$, 535[M+H+NBA]$^+$).

The starting materials can be prepared as follows:

Tetrahydropyranyl-D-2-cyclopentyl-2-hydroxyacetyl--L-proline (THP-D-cPga-Pro)

Step A: Resolution of O-acetyl-DL-2-cyclopentyl-2--hydroxyacetic acid with acylase enzyme

0.72 g (5.0 mmole) of DL-2-cyclopentyl-2-hydroxyacetic acid [M. Robba and Y. Le Guen, Chim. Therap. **1966**, 238; Chem. Abst. **66**, 18633f (1967)] is acetylated and hydrolyzed with acylase I by the process described in Example 1, Steps A and B, using proportional amounts of reagents and solvents. The resulting mixture of L-2-cyclopentyl-2--hydroxyacetic acid [R_f(14) = 0.35] and O-acetyl-D-2--cyclopentyl-2-hydroxyacetic acid [R_f(14) = 0.52] is separated and the latter one is transformed by the process described in Example 1, Step C, using proportional amounts of reagents and solvents, into D-2-cyclopentyl-2-hydroxy-acetic acid. Yield:

a) 0.2 g (1.39 mmole) of L-2-cyclopentyl-2-hydroxyacetic acid, m. p. 108-109°C;

$[\alpha]_D$ = -15.95° (c = 1, methanol); optical purity 99 % (chiral HPLC), and

b) 0.16 g (1.1 mmole) of D-2-cyclopentyl-2-hydroxyacetic acid, m. p. 106°C;
$[\alpha]_D$ = +15.5° (c = 1, methanol); optical purity 98 % (chiral HPLC).

Step B: D-2-Cyclopentyl-2-hydroxyacetic acid (resolution of DL-compound with D-tyrosine hydrazide)

1.44 g (10 mmole) of DL-2-cyclopentyl-2-hydroxyacetic acid [M. Robba and Y. Le Guen, Chim. Therap. **1966**, 238; Chem. Abst. **66**, 18633f (1967)] and 1.97 g (10 mmole) of D-tyrosine hydrazide are suspended in 50 mL of anhydrous ethanol and refluxed up to full dissolution. Then the solution is left to cool to room temperature and kept in the refrigerator overnight. The crystals formed are filtered, washed with 2 x 2 mL of cold, anhydrous ethanol and dried at reduced pressure. The obtained 2.14 g (6.3 mmole) of diastereomer salt is recrystallyzed twice from 40 mL of anhydrous ethanol. The resulting 1.54 g (4.53 mmole) of diastereomer salt is dissolved in 10 mL of diethyl ether and 5 mL of 1 M potassium hydrogen sulfate solution. The diethyl ether phase is washed to neutrality with water, dried over anhydrous sodium sulfate and evaporated at 2.0 - 2.5 kPa. The crystalline residue is worked up with petroleum ether, filtered and air-dried.
Yield 0.56 g (3.88 mmole, 76.7 %) of D-2-cyclopentyl-2--hydroxyacetic acid, m. p. 109-110°C;
$[\alpha]_D$ = +14.8° (c = 1, methanol) and -30° (c = 1, acetic acid).
Optical purity 98 % (chiral HPLC).

Analysis for $C_7H_{12}O_3$ (144.17)

Calculated: C% = 58.31; H% = 8.39;

Found: C% = 58.56; H% = 8.50.

Step C: D-2-Cyclopentyl-2-hydroxyacetic acid benzyl ester

0.47 g (3.2 mmole) of D-2-cyclopentyl-2-hydroxyacetic acid (Example 2, Step B) is transformed by the process described in Example 1, Step E, using proportional amounts of reagents and solvents.

Yield 0.74 g (95 %) of the aimed product in oil form [R_f(12) = 0.20].

Step D: Tetrahydropyranyl-D-2-cyclopentyl-2-hydroxy-acetic acid benzyl ester

0.74 g (3.2 mmole) of D-2-cyclopentyl-2-hydroxyacetic acid benzyl ester (Example 2, Step C) is transformed by the process described in Example 1, Step F, using proportional amounts of reagents and solvents. The resulting evaporation residue is considered 2.75 mmole of the aimed product R_f(12) = 0.27 - 0.36.

Step E: Tetrahydropyranyl-D-2-cyclopentyl-2-hydroxy-acetic acid

2.75 mmole of tetrahydropyranyl-D-2-cyclopentyl-2--hydroxyacetic acid benzyl ester (Example 2, Step D) is

transformed by the process described in Example 1, Step G, using proportional amounts of reagents and solvents.
Yield 0.6 g (2.6 mmole, 95 %) of the aimed product [$R_f(1) = 0.25$].

Step F: Tetrahydropyranyl-D-2-cyclopentyl-2-hydroxy-acetyl-L-proline benzyl ester

2.6 mmole of tetrahydropyranyl-D-2-cyclopentyl-2--hydroxyacetic acid (Example 2, Step E) and 0.62 g (2.6 mmole) of L-proline benzyl ester hydrochloride are condensed by the process described in Example 1, Step H, using proportional amounts of reagents and solvents.
Yield 0.97 g (90 %) of the aimed product in oil form.
$R_f(1) = 0.7$.

Step G: Tetrahydropyranyl-D-2-cyclopentyl-2-hydroxy-acetyl-L-proline

0.97 g (2.3 mmole) of tetrahydropyranyl-D-2-cyclo-pentyl-2-hydroxyacetyl-L-proline benzyl ester (Example 1, Step F) is transformed by the process described in Example 1, Step I, using proportional amounts of reagents and solvents.
Yield 0.71 g (89 %) of the aimed product in oil form [$R_f(9) = 0.5$], a major portion of which is used directly in Step 1 of the Example and a small portion is converted to a crystalline salt as follows.

0.03 g (0.1 mmole) of tetrahydropyranyl-D-2-cyclopentyl-2-hydroxyacetyl-L-proline (oil) is dissolved in 5 mL of diethyl ether and 0.018 mL (0.1 mmole) of dicyclohexylamine is added. The crystals formed are filtered, washed with diethyl ether and dried at reduced pressure. Yield 0.048 g (95 %) of tetrahydropyranyl-D-2-cyclopentyl--2-hydroxyacetyl-L-proline dicyclohexylammonium salt.

M. p.: 145 -147°C

Analysis for $C_{29}H_{50}N_2O_5$ (506.73)

Calculated: C% = 68.73; H% = 9.95; N% = 5.52;

Found: C% = 67.25; H% = 9.94; N% = 6.20.

The FAB mass spectrum confirms the assumed structure: (507[M+H+DCHA]$^+$).

Methods

Method M1

Preparation of plasma clot

a) Platelet-rich plasma (PRP) is prepared by centrifuging a 9:1 mixture of human blood and 3.8 % aqueous sodium citrate solution (1/2) for 5 minutes at 240 x g.

b) 200 µL of PRP are placed in each reaction vessel, 80 µL of 40 mM calcium chloride solution is added and the mixture is left to stand at room temperature for one hour. The plasma clot formed is rinsed mildly with 6 x 2 ml of 0.9 % sodium chloride solution followed by 5 minute sedimentation to remove the dissolved enzymes (factor Xa and thrombin). The thrombin content of the washings is assayed as follows.

c) To 400 µL of washing liquid 100 µL of 1 mM Tos-Gly-Pro--Arg-pNA substrate solution is added and the mixture is

incubated for 30 minutes at 37°C, then the reaction is stopped by the addition of 100 µL of 50 % acetic acid. 150 µL portions of this mixture are transferred into the wells of the 96-well microtiter plate and the extinction is measured at 405 nm (ELISA READER SLT Laborinstrument GmbH, Austria). When the washing is successful, the extinction is less than 5 % of the control.

Method M2

Assay of the inhibition of factor Xa entrapped in plasma clot

a) 0.1 - 1.0 - 10 and 100 µg/mL solutions of the peptide inhibitor are prepared with 0.1 M Tris buffer containing 0.02 % human albumin at pH 8.5.

b) After draining the washing liquid, 400 µL of the peptide solution (3 parallel samples for each concentration) and 400 µL of buffer, serving as control, are placed on the clot (Method M1) and the mixture is incubated for 5 minutes at 37°C. Thereafter 100 µL of 2 mM Moc-D-Chg-Gly-Arg-pNA substrate solution is added and incubation at 37°C is continued for further 30 minutes, then the reaction is stopped by the addition of 100 µL of 50 % acetic acid.

c) 150 µL portions of each reaction mixture are placed into the wells of a 96-well microtiter plate and the extinction is determined at 405 nm (ELISA READER SLT Laborinstrument GmbH, Austria). The peptide concentration (IC_{50}) required for 50 % inhibition is determined graphically from the mean extinction values related to the controls.

Method M3

Assay of the inhibition of thrombin entrapped in plasma clot

a) Peptide inhibitor solutions are prepared by the process described in Method M2, point a).

b) After draining the washing liquid, 400 µL of the peptide solution (3 parallel samples for each concentration) and 400 µL of buffer, serving as control, are placed on the clot (Method M1) and the mixture is incubated for 5 minutes at 37°C. Thereafter 100 µL of 1 mM Tos-Gly-Pro-Arg-pNA substrate solution is added and incubation at 37°C is continued for further 30 minutes, then the reaction is stopped by the addition of 100 µL of 50 % acetic acid. The operation is continued as described in Method M2, point c).

Method M4

Preparation of fibrin-gel

a) 200 µL of 6 mg/mL human fibrinogen (SIGMA), 25 µL of 25 NIH U/mL human thrombin (SIGMA) and 40 µL of 100 mM calcium chloride solution are placed in each reaction vessel and the mixtures are stored for one hour at 20 - 22°C. The fibrin formed is rinsed mildly with 3 x 2 ml of isotonic saline solution followed by sedimentation to remove the thrombin content of the solution. The efficacy of rinsing is controlled by the process described in Method M1, point c).

Method M5

Assay of the inhibition of thrombin bound to fibrin-gel

a) 0.1 - 1.0 - 10 and 100 µg/ml solutions are prepared with Hepes/NaCl buffer (0.01 M Hepes and 0.1 M sodium chloride, pH = 7.4).

b) Thereafter the procedure described in Method M3, point b) is followed.

Method M6

Assay of the inhibition of factor Xa in solution on a 96-well microtitre plate

a) 1.3 U/mL factor Xa (human, SIGMA), 0.1 - 1.0 - 10 and 100 µg/mL solutions in phosphate buffer (0.1 M sodium phosphate and 0.05 M sodium chloride, pH 7.4) of the peptide inhibitors and 0.33 nM solutions of the substrate Bz-Ile-Glu-Gly-Arg-pNA in distilled water are used.

b) Three reaction mixtures are prepared from the control and each peptide solution. 30 µL each of the peptide and the buffer, serving as control, 30 µL factor Xa, 90 µL buffer and 150 µL substrate are placed in the wells of the plate, then after 10 minutes the extinction values are read at 405 nm. The procedure is continued as described in Method M2, point c).

What we claim is

1. New peptide aldehyde derivatives of general formula (I),

D-Xaa-Pro-Arg-H (I)

wherein

Xaa represents a 2-cycloheptyl-2-hydroxyacetyl or 2-cyclopentyl-2-hydroxyacetyl group,

Pro represents an L-prolyl residue and

Arg represents an L-arginyl residue,

and their acid-addition salts formed with organic or inorganic acids.

2. D-2-Cycloheptyl-2-hydroxyacetyl-L-prolyl-L--arginine aldehyde and its acid-addition salts.

3. D-2-Cycloheptyl-2-hydroxyacetyl-L-prolyl-L--arginine aldehyde hemisulfate.

4. D-2-Cyclopentyl-2-hydroxyacetyl-L-prolyl-L--arginine aldehyde and its acid-addition salts.

5. D-2-cyclopentyl-2-hydroxyacetyl-L-prolyl-L--arginine aldehyde hemisulfate.

6. A pharmaceutical composition which comprises as active ingredient at least one compound of general formula (I), wherein Xaa, Pro and Arg are as defined in claim 1, or a pharmaceutically acceptable acid-addition salt thereof in admixture with solvents, diluents, carriers and/or additives commonly used in the pharmaceutical industry.

7. A compound as claimed in any of claims 1 to 6 for use as therapeutic agent.

8. A compound as claimed in any of claims 1 to 6 for use as antithrombotic therapeutic agent.

INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/HU 97/00027

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07D207/09 A61K38/05 A61K38/36

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LETT.PEPT.SCI., vol. 2, no. 3/4, 1995, pages 147-154, XP002041031 JONES D.M. ET AL.: "Design and synthesis of thrombin inhibitors" whole document ---	1-8
A	J.MED.CHEM., vol. 38, 1995, pages 4446-4453, XP002041032 SHUMAN R.T. ET AL.: "Structure-Activity Study of Tripeptide Thrombin Inhibitors Using Alpha-Alkyl Amino Acids and other Conformationally Constrained Amino Acids" whole document --- -/--	1-8

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 September 1997	02. 10. 97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Kronester-Frei, A

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INTERNATIONAL SEARCH REPORT

Intern. nal Application No
PCT/HU 97/00027

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIOORGANIC & MEDICAL CHEMISTRY, vol. 3, no. 8, 1995, pages 1079-1089, XP002041033 BAJUSZ S. ET AL.: "Active Site-Directed Thrombin Inhibitors: Alpha-Hydroxyacyl-prolyl-arginals. New Orally active Stble Analogs of D-Phe-Pro-Arg-H" cited in the application Tables	1-8

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